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Mutation of E1 glycoprotein of classical swine fever virus affects viral virulence in swine

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Abstract

Transposon linker insertion mutagenesis of a full-length infectious clone (IC) (pBIC) of the pathogenic classical swine fever virus (CSFV) strain Brescia was used to identify genetic determinants of CSFV virulence and host range. Here, we characterize a virus mutant, RB-C22v, possessing a 19-residue insertion at the carboxyl terminus of E1 glycoprotein. Although RB-C22v exhibited normal growth characteristics in primary porcine macrophage cell cultures, the major target cell of CSFV in vivo, it was markedly attenuated in swine. All RB-C22v-infected pigs survived infection remaining clinically normal in contrast to the 100% mortality observed for BICv-infected animals. Comparative pathogenesis studies demonstrated a delay in RB-C22v spread to, and decreased replication in the tonsils, a 10^2 to 10^7 $\log_{10}$ reduction in virus titers in lymphoid tissues and blood, and an overall delay in generalization of infection relative to BICv. Notably, RB-C22v-infected animals were protected from clinical disease when challenged with pathogenic BICv at 3, 5, 7, and 21 days post-RB-C22v inoculation. Viremia, viral replication in tissues, and oronasal shedding were reduced in animals challenged at 7 and 21 DPI. Notably BICv-specific RNA was not detected in tonsils of challenged animals. These results indicate that a carboxyl-terminal domain of E1 glycoprotein affects virulence of CSFV in swine, and they demonstrate that mutation of this domain provides the basis for a rationally designed and efficacious live-attenuated CSF vaccine.

Keywords: CSFV; Virulence; Pathogenesis; Attenuation; Vaccine; Protection; Glycoproteins; Transposon; Mutagenesis

Introduction

Classical swine fever virus (CSFV) is the causative agent of classical swine fever (CSF), a highly contagious and significant pathogen of swine (van Oirschot, 1986). CSFV, a member of the genus *Pestivirus* of the family Flaviviridae (Wengler et al., 1995), is a small enveloped virus with a single-stranded, 12.5 kb RNA genome of positive polarity that is translated as a single polyprotein (Rice, 1996). The approximately 4000 amino acid polyprotein, once processed, gives rise to the following 11 to 12 final cleavage products: NH_2-Npro-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Rice, 1996). Protein C and glycoproteins E^{rns}, E1, and E2 represent structural components of the virion (Thiel et al., 1991). While E1 and E2 are anchored to the envelope by their carboxyl termini, E^{rns} appears to be covalently associated with the other envelope glycoproteins (Weiland et al., 1990, 1999). E^{rns} and E2 are present as disulfide-linked homodimers on the virion surface,

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and E2 is also present as a heterodimer with E1 (Weiland et al., 1990). Although E^{rns} and E2 have been shown to function in viral pathogenesis, virus attachment to target cell, and the induction of a protective immune responses (Hulst et al., 1993, 1998; Hulst and Moormann, 1997; Meyers et al., 1999; Risatti et al., 2005; Ruggli et al., 1996; van Gennip et al., 2000, 2004; van Zijl et al., 1991; Weiland et al., 1990, Widjojoatmodjo et al., 2000), the function of E1 is less well understood. A recent study has shown that E1 and E2 are sufficient to mediate entry of pseudotyped retrovirus into swine cells (Wang et al., 2004).

Although highly virulent strains of CSFV in pigs cause severe disease characterized by high morbidity and mortality, strains of moderate to low virulence that induce prolonged chronic disease have been isolated (van Oirschot, 1986). In addition, avirulent strains of CSFV obtained through cell culture passage have provided the basis for effective live-attenuated vaccines (LAV) for CSF, exhibiting the ability to infect animals and induce protective immunity without inducing clinical disease (Terpstra et al., 1990). Specific genetic determinants are likely responsible for these different phenotypes; however, the genetic basis of CSFV virulence remains poorly understood (Aynaud, 1988; Bironi et al., 1987). Notably, recent development of cDNA infectious clones (IC) of attenuated and virulent CSFV strains is enhancing understanding of molecular mechanisms of viral replication and pathogenesis and allowing rational design and engineering of improved LAV for CSF (Mayer et al., 2004; Meyers et al., 1999; Moormann et al., 1996; Moser et al., 2001; Risatti et al., 2005; Ruggli et al., 1996; van Gennip et al., 2000, 2004; Widjojoatmodjo et al., 2000).

Using CSFV IC, several viral proteins as having roles in viral virulence, including E^{rns}, N^{pro}, and E2, have been identified. Depending on the specific E^{rns} mutations engineered into virulent CSFV strain Alfort/187, variable degrees of attenuation were observed when mutants were inoculated in swine (Meyers et al., 1999). Complete deletion of N^{pro} from Alfort/187 and Eystrup strains abrogates their virulence for swine (Mayer et al., 2004). Recently, CSFV IC have been used to demonstrate that E2 harbors genetic determinants of virulence (Risatti et al., 2005; van Gennip et al., 2004).

In efforts to identify additional virulence and host range determinants of CSFV, we used transposon linker insertion mutagenesis (TLIM) (Hallet et al., 1997; Hobom et al., 2000) to mutate an IC of the pathogenic strain Brescia (pBIC) (Risatti et al., 2005). A viral mutant, RB-C22v, with a 57 nucleotide insertion at genomic position 2429 was obtained and found to be highly attenuated in pigs. RB-C22v exhibited decreased replication in tonsils, limited generalization of infection, and a significant reduction of virus shedding following infection of swine. Notably, RB-C22v-infected pigs challenged with pathogenic BICv at 3, 5, 7, and 21 days post-RB-C22v inoculation (DPI) were protected from clinical disease. These data indicate that mutation of a carboxyl-terminal domain of the E1 glycoprotein markedly affects swine virulence. Identification of this and other CSFV virulence and host range determinants will permit the rational design of CSF LAV with improved safety, efficacy, and utility.

Results

Generation of attenuated BICv mutant virus RB-C22v

Mutants of BICv were obtained using transposon linker insertion mutagenesis, and derivative viruses were rescued

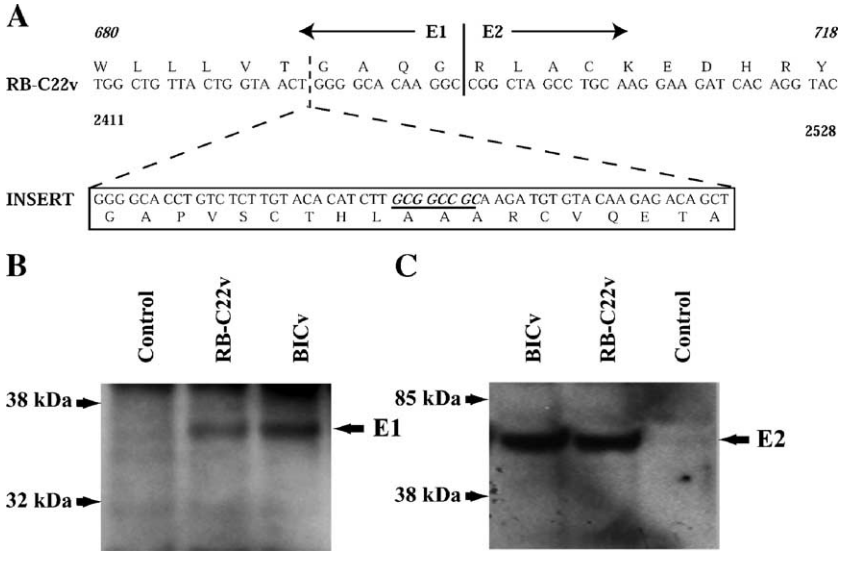

Fig. 1. RB-C22 virus was generated by transposon linker insertion mutagenesis of a full-length infectious clone (IC) (pBIC) of the pathogenic classical swine fever virus (CSFV) strain Brescia (A). A 57-nucleotide in-frame insertion encoding 19 amino acids (box) is located at the carboxyl terminus of RB-C22v envelope glycoprotein E1, four residues upstream (dotted line) of the cleavage site between E1 and envelope glycoprotein E2 (solid line). Numbers in bold indicate nucleotide position, numbers in bold italics indicate amino acid residue position. Underlined residues in bold italics represent the *Not*I restriction site. The analysis of CSFV glycoproteins E1 and E2 expression in infected SK6 cells was done by Western immunoblot (B and C). SK6 monolayers were infected (MOI = 1) with RB-C22v, BICv, or mock-infected (control) and harvested at 48 h post-infection. Cell lysates were run under denaturing conditions in 12% SDS-PAGE. CSFV E1 (B) was detected with an E1 specific rabbit antiserum, and CSFV E2 (C) was detected with mAb WH303.

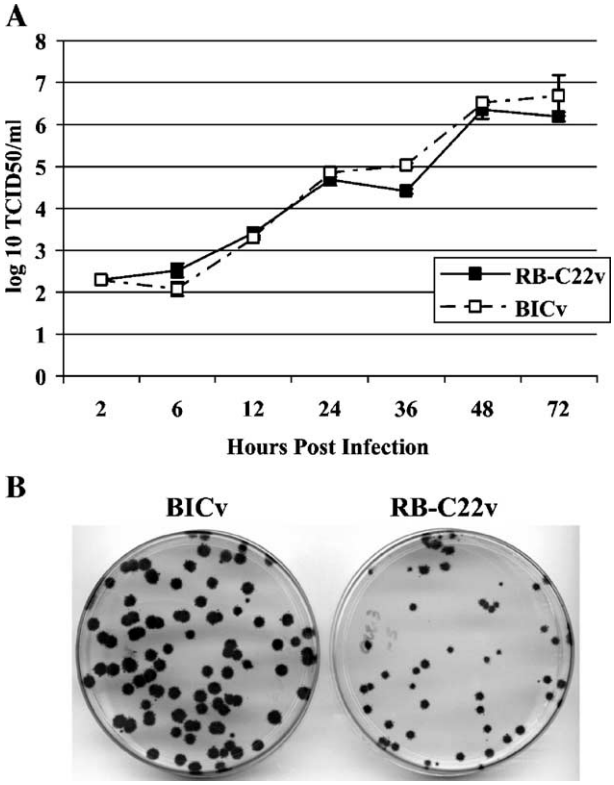

Fig. 2. (A) Multistep growth curve of BICv and RB-C22v on swine macrophage cell cultures. Primary swine macrophage cell cultures were infected (MOI of 0.1) with CSFV BICv (open squares) or RB-C22v (filled squares). At times post-infection, samples were collected and titrated for virus yield. Data are means and standard errors of three independent experiments. (B) Plaque formation of CSFV BICv and RB-C22v on SK6 cell cultures. Cell cultures were infected with approximately 75 $TCID_{50}$ of CSFV BICv or RB-C22v, overlaid with 0.5% agarose, incubated at 37 °C for 3 days, fixed with 50% (v/v) ethanol/acetone, and stained by immunohistochemistry as described in Materials and methods.

from SK6 cells using in vitro transcription and RNA transfection. One viral mutant, RB-C22v, exhibited an altered virulence phenotype in swine (data not shown). RB-C22v contained at genomic position 2429, a 57-nucleotide in-frame insertion encoding 19 amino acids inserted at the carboxyl terminus of envelope glycoprotein E1, four residues upstream of the cleavage site between E1 and envelope glycoprotein E2 (Fig. 1A). The complete nucleotide sequence of RB-C22v was obtained and compared with that of BICv. Complete nucleotide sequences for both viruses were identical except for the 57-nucleotide insertion in RB-C22v (data not shown). Relative electrophoretic mobility in Western blot did not show noticeable differences between E1 or E2 in RB-C22v-infected and BICv-infected SK6 cells (Figs. 1B and C).

Mutation of E1 does not affect growth of RB-C22v in porcine macrophage cell cultures

Growth characteristics of RB-C22v and BICv were compared in a multistep growth curve by infecting primary swine macrophage cell cultures (MOI = 0.1) and determining titers for both at times post-infection. Growth kinetics of RB-C22v and BICv were indistinguishable (Fig. 2A). Notably, the plaque size of RB-C22v on SK6 cell cultures was reduced by more than 50% relative to plaques formed by BICv (Fig. 2B).

E1 mutation affects CSFV virulence in pigs

To examine the effect of the RB-C22v E1 mutation on pig virulence, pigs were inoculated IN with 10^5 $TCID_{50}$ of RB-C22v or BICv, and the disease course was monitored. The disease pattern observed for RB-C22v-infected animals contrasted markedly with that seen in BICv-infected animals. All RB-C22v-infected animals survived and remained clinically normal following infection (with the exception of a transient hyperthermia in half of the infected animals), whereas animals infected with BICv exhibited 100% mortality and presented clinical signs of CSF 3 to 7 DPI, with symptoms progressing until death (Table 1). Blood lymphocyte and platelet counts dropped drastically in BICv-infected animals at 4 to 6 DPI and remained low until death, while a transient and much less dramatic effect was observed in the RB-C22v-infected animals (Fig. 3). Both mean and maximum viremia titers in RB-C22v-infected animals were significantly reduced (10^2 to 10^7 $\log_{10}$) relative to those in control animals during the acute disease period (Table 2). Additionally, RB-C22v titers in nasal swabs and tonsil scrapings were significantly decreased (10^1 to 10^3 $\log_{10}$) (Table 2). The complete nucleotide sequence of RB-C22v, isolated at 6 DPI from the tonsils of an infected pig, was identical to RB-C22v stock virus used for animal inoculations (data not shown). These results demonstrate that RB-C22v is markedly attenuated for swine.

For detailed comparison of RB-C22v and BICv pathogenesis, randomly selected animals were euthanized at 2, 4, 6, 9, 11, and 16 DPI, and tissue samples were collected for virus titration, histopathological, and immunohistochemical analysis. Virus titers in tissues are shown in Table 2. RB-C22v replication in tonsils was significantly decreased (between 10^2 and 10^7 $\log_{10}$, depending on DPI) relative to BICv. At 4–6 DPI, RB-C22v was present in the draining submandibular lymph node but at titers 100- to 1000-fold lower than those observed in BICv-infected animals. Generalization of infection occurred in both RB-C22v and BICv-infected animals by 4

Table 1
Swine survival and fever response following infection with RB-C22v or BICv

Virus	Number of survivors/total	Mean time to death; days (SD)	Mean time of fever onset; days (SD)	Mean time duration of fever; days (SD)	Max daily temperature; average (SD)
RB-C22v	8/8	–	5.5 (1.0)	0.7 (0.8)	104.1 (0.6)
BICv	0/5	9.12 (3.09)	4.4 (0.8)	4.6 (0.8)	106.1 (0.5)

SD: standard deviation.

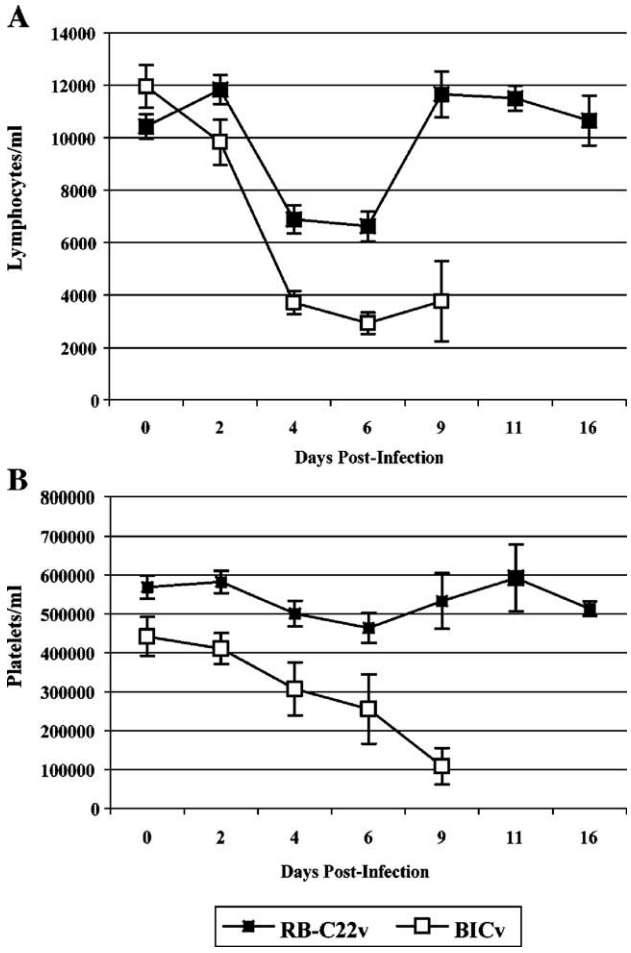

Fig. 3. Cell counts of (A) peripheral blood lymphocytes and (B) platelets in pigs infected with BICv (empty squares) or RB-C22v (filled squares). Each point represents the mean and standard error of 3 or more animals.

DPI; however, from 4 DPI onward, RB-C22v titers in spleen, kidney, and mesenteric lymph node were significantly lower (approximately 10^2 to 10^3 $\log_{10}$) than values observed for BICv-infected animals. Interestingly, central nervous system (CNS) involvement in RB-C22v-infected animals was markedly reduced relative to that seen in BICv-infected animals (Table 2). Overall, RB-C22v infection resulted in reduced generalization of infection and significantly lower virus titers in tissues.

Histological lesions in pigs inoculated with BICv were observed as early as 4 DPI. These included tonsilar and submandibular lymphoid necrosis and depletion, which progressed to abscess formation by 10 DPI. Additionally, between 6 and 10 DPI, there was moderate to marked histiocytic hyperplasia and reticuloendothelial cell proliferation (Fig. 4). RB-C22v-infected pigs showed similar pathology of the tonsil and regional submandibular lymph node, but the degree was markedly reduced, with a minimal to mild lymphoid necrosis and only a mild histiocytosis. Notably, lesions in RB-C22v-infected animals began to resolve with evidence of lymphoid hyperplasia by 11 DPI. BICv-infected spleen was characterized by moderate to severe lymphoid follicular depletion, necrosis, and mild to moderate histiocytosis, while, in RB-C22v-infected pigs, there were negligible changes in the spleen with minimal lympholysis and depletion (Fig. 4). As early as 6 DPI, BICv-infected pigs had a moderate to marked meningoencephalitis with vasculitis and prominent perivascular lymphohistiocytic cuffs, and, by 10 DPI, cerebral and cerebellar necrosis were associated with severe vasculitis and thrombosis. In contrast, there was minimal pathology in CNS of the RB-C22v-infected pigs, with only 7 of 18 animals exhibiting rare perivascular cuffs of a few lymphocytes and macrophages and foci of gliosis.

The presence of CSFV antigen in tissues of infected animals was consistent with the degree and distribution of lesions and virus tissue titers. In BICv-infected pigs, there was antigen in superficial and cryptic tonsilar epithelial cells and tonsilar endothelium by 2 DPI and in tonsilar macrophages and lymphoid follicles with moderate to marked immunoreactivity by 4 DPI (Fig. 4). Similar immunoreactivity was observed in mandibular lymph node and the spleen, which included macrophages and endothelium. In the brain of the BICv-infected animals, there was CSFV antigen labeling by 2 DPI with moderate to marked immunoreac-

Table 2
Virus titers in clinical samples and tissues following infection with BICv or RB-C22v[a]

Samples	BICv ($\log_{10}$ $TCID_{50}$/ml) DPI						RB-C22v ($\log_{10}$ $TCID_{50}$/ml) DPI					
	2	4	6	9	11	16	2	4	6	9	11	16
Nasal swabs	Neg	Neg	2.30 (1.15)	4.47 (1.15)	ND	ND	Neg	Neg	1.85 (0.19)	1.84 (0.29)	Neg	Neg
Tonsil scrapings	Neg	2.79 (0.26)	2.00 (0.08)	4.47 (0.19)	ND	ND	Neg	1.92 (0.03)	Neg	Neg	Neg	Neg
Blood	Neg	3.11 (0.34)	6.50 (0.27)	9.02 (0.28)	ND	ND	Neg	2.09 (0.32)	2.81 (0.75)	1.84 (0.08)	Neg	Neg
Tonsil	1.40 (1.40)	5.55 (0.75)	6.80 (0.17)	7.71 (0.09)	7.80 (0.00)	ND	Neg	3.19 (0.53)	5.58 (0.23)	1.97 (0.17)	1.76 (0.39)	1.89 (0.12)
Mandibular lymph node	Neg	4.05 (0.08)	5.55 (0.25)	7.47 (0.34)	7.80 (0.00)	ND	Neg	1.86 (0.10)	2.58 (0.92)	Neg	Neg	Neg
Mesenteric lymph node	Neg	2.14 (0.47)	5.64 (0.17)	7.55 (0.25)	7.80 (0.00)	ND	Neg	2.14 (0.16)	2.58 (0.24)	Neg	Neg	Neg
Spleen	Neg	2.14 (1.17)	5.97 (0.00)	7.47 (0.34)	7.80 (0.00)	ND	Neg	Neg	1.97 (0.00)	1.86 (0.10)	Neg	Neg
Kidney	2.47 (0.67)	1.97 (0.00)	4.47 (0.17)	7.22 (0.42)	7.47 (0.00)	ND	Neg	Neg	Neg	Neg	Neg	Neg
Bone marrow	Neg	3.05 (0.08)	7.63 (0.00)	7.87 (0.07)	6.99 (0.00)	≤1.80	Neg	1.97 (0.17)	3.08 (0.78)	1.97 (0.17)	Neg	Neg
Brain	Neg	Neg	5.05 (1.08)	4.64 (0.84)	6.80 (0.00)	≤1.80	Neg	Neg	Neg	Neg	Neg	Neg

[a] Titers expressed as $TCID_{50}$/ml. DPI, days post-infection. ND, not determined. Numbers in parenthesis indicate standard deviation. Neg: ≤1.80 $TCID_{50}$/ml.

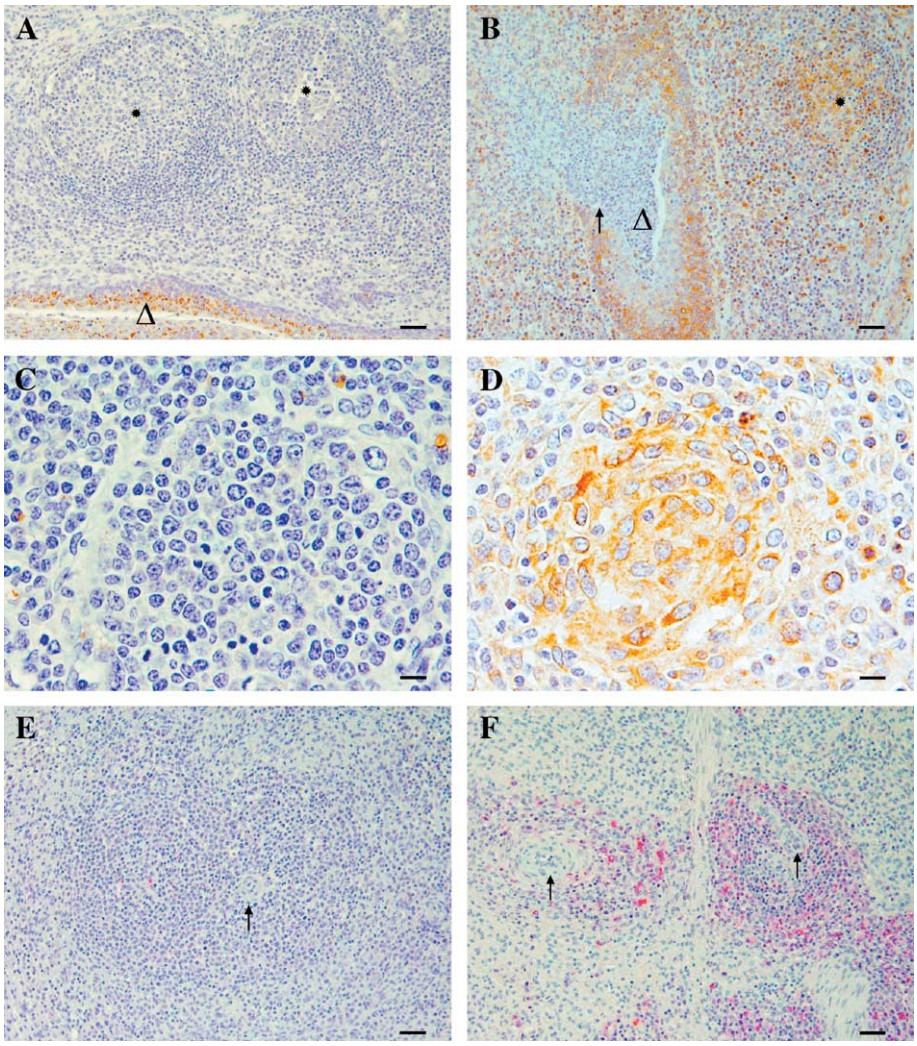

Fig. 4. Detection of CSFV antigens in lymphoid organs of pigs infected with BICv or RB-C22v viruses by immunohistochemistry. Tonsils at 9 DPI (A and B) and 11 DPI (C and D) in animals infected with RB-C22v (A and C) or BICv (B and D). Note hyperplastic lymphoid follicles (*) and abundant immunoreactivity in the crypt epithelium (Δ), but not in lymphoid follicles, in RB-C22v-infected animals versus abundant immunoreactivity both in lymphoid follicles and crypt epithelium, along with depletion of the lymphoid follicles, in animals infected with BICv. Also note at 11 DPI the hyperplasic periarteriolar lymphoid sheaths (E) (→) and absence of immunoreactivity in the spleen of RB-C22v-infected animals (E) versus depleted lymphoid follicles and abundant immunoreactivity in the periarteriolar area of BICv-infected animals (F).

tivity by 6 DPI in vascular endothelium, perivascular macrophages, and in glial cells, with the latter more pronounced at 10 DPI. In contrast, the highest immunoreactivity in RB-C22v-infected pigs was observed in tonsils, with a significant reduction of staining in the submandibular lymph node, spleen, and brain. In the brain, minimal

Table 3
Swine survival and fever response of RB-C22v-infected animals following challenge with BICv

Challenge DPI[a]	Number of Survivors/total	Mean time to death; days (SD)[b]	Mean time of fever onset; days (SD)	Mean time duration of fever; days (SD)	Max daily temperature; average (SD)
1	1/3	12.6 (3.5)	4.0 (0.0)	7.0 (4.3)	106.8 (0.3)
3	3/3	–	4.0[c]	1.0	104.5 (0.7)
5	3/3	–	3.0[c]	1.0	104.1 (0.6)
7	8/8	–	None	–	103.7 (0.3)
21	8/8	–	None	–	102.4 (0.3)
Control[d]	0/8	9.0 (2.8)	3.6 (1.7)	4.4 (1.3)	106.1 (0.5)

[a] Days post-infection with RB-C22v.
[b] SD, standard deviation.
[c] Observed on a single animal.
[d] Control, no RB-C22v infection.

reactivity was observed in only 4 of 18 RB-C22v-infected animals.

Overall, the data indicate that mutation of glycoprotein E1 affects CSFV virulence for swine. RB-C22v infection is characterized by an absence of clinical disease, decreased viral replication in tonsils, limited generalization of infection, and dramatically reduced virus shedding.

RB-C22v infection protects pigs against challenge with pathogenic BICv

The ability of RB-C22v to induce protection against BICv challenge was evaluated. At 1, 3, 5, 7, and 21 DPI with RB-C22v, pigs were challenged with 10^5 $TCID_{50}$ of pathogenic BICv.

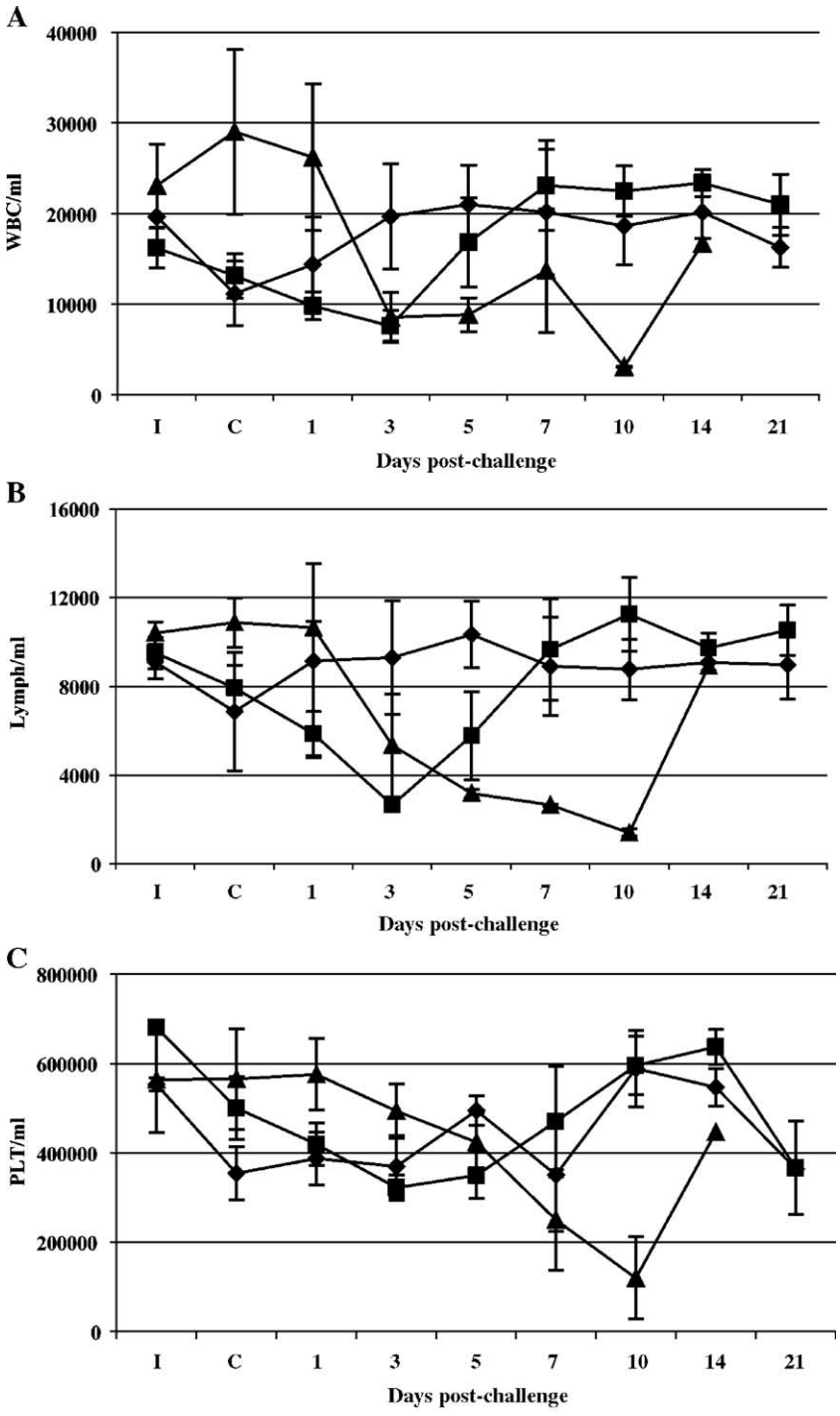

Fig. 5. (A) Peripheral white blood cell, (B) lymphocyte, and (C) platelet counts in pigs infected with RB-C22v and challenged at 1 DPI (triangles), 3 DPI (squares), or 5 DPI (diamonds) with BICv. Counts are expressed as numbers/ml and represent the mean of all individuals in the group, with error bars indicating standard error. I, infection with RB-C22v. C, challenge with BICv.

Table 4
Virus titers and RT-PCR detection of viral RNA in clinical samples obtained from RB-C22v-infected and uninfected control animals challenged with BICv at 7 DPI

Days post-challenge	RB-C22v-infected (n = 8)					Non-infected (n = 4)				
	Tonsil scrapings		Nasal Swabs		Viremia	Tonsil scrapings		Nasal Swabs		Viremia
	Titer[a]	RNA[b]	Titer	RNA	Titer	Titer	RNA	Titer	RNA	Titer
5	Neg	–	Neg	–	Neg	2.4 (1.6)	+	3.1 (0.2)	+	5.6 (0.8)
7	Neg	–	Neg	–	Neg	5.4 (0.8)	+	4.8 (0.0)	+	7.5 (0.7)
9	Neg	–	Neg	–	Neg	4.6 (0.8)	D[c]	6.0 (0.0)	+	ND[d]
12	Neg	–	Neg	–	Neg	D	D	D	D	D
16	Neg	–	Neg	–	Neg	D	D	D	D	D
19	Neg	–	Neg	–	Neg	D	D	D	D	D

Neg: ≤1.80 $TCID_{50}$/ml.
[a] Virus titers expressed as $\log_{10}$ $TCID_{50}$/ml and standard deviation shown in parenthesis. –, negative.
[b] CSFV viral RNA present (+) or absent (–) as detected by real-time RT-PCR (23).
[c] D, dead animals.
[d] ND, not determined.

Pigs challenged with BICv 1 day after RB-C22v infection were partially protected. Animals developed hematological changes and clinical signs, including leukopenia, lymphocytopenia, and thrombocytopenia by 3 days post-challenge (DPC) with fever, anorexia, and depression evident by 5 DPC (Table 3 and Fig. 5). Of these three animals, two pigs died (9 and 13 DPC). Clinical signs and body temperature of the surviving animal returned to normal by 12 DPC (Table 3). Control pigs receiving only BICv challenge developed anorexia, depression, and fever by 4 DPC, a marked reduction of leukocytes, lymphocytes, and platelets by day 5 DPC, and died or were euthanized in extremis by 12 DPC (Table 3).

Notably, complete protection against BICv-induced clinical disease was induced 3 days post-RB-C22v infection. All pigs survived infection and remained clinically normal, with only a single animal presenting a transient fever at 4 DPC (Table 3). A transient drop in hematological values was observed between 1 and 5 DPC (Fig. 5). Similarly, pigs challenged at 5, 7, and 21 days post-RB-C22v infection remained clinically normal, with the exception of a transient fever in 2 of 19 animals (Table 3, Fig. 5, and data not shown).

Virus was not detected in blood or oronasal swabs samples of RB-C22v-infected pigs challenged at 7 or 21 DPI nor could viral RNA be detected in nasal and tonsil samples by RT-PCR (Table 4 and data not shown). Viremia was observed in control animals by 5 DPC, with virus titers remaining high (10^7 $TCID_{50}$/ml by 7 DPC) until death (Table 4). In addition, virus was titrated from oronasal swabs of control animals by 5 DPC (Table 4), reaching titers of 10^6 $TCID_{50}$/ml by 9 DPC.

Antibody responses to CSFV were measured by ELISA and serum neutralization. All RB-C22v-infected animals developed E2-specific antibodies by 14 DPI and significantly higher levels by the time of challenge at 21 DPI as detected with both CSFV E2-specific ELISA tests. Neutralizing antibodies were detected in 7 of 8 RB-C22v-infected pigs as early as 14 DPI ($\log_{10}$ 2.02, SD = 0.12) and in serum from all animals when challenged at 21 DPI ($\log_{10}$ = 2.44, SD = 0.14). Notably, RB-C22v-infected animals at 7 DPI were efficiently protected from BICv challenge despite lacking detectable antibodies against CSFV at challenge (Table 3).

An RT-PCR assay that differentiates RB-C22v from BICv was used to identify virus present in tonsils of animals challenged at 1 or 3 DPI with RB-C22v (see Materials and methods). Genomic RNA from both viruses was detected in equivalent amounts in tonsils of animals challenged at 1 DPI (Table 5). Animals in this group developed disease and were not protected (Table 3). However, RB-C22v was the only virus detected in tonsils of protected animals challenged at 3 DPI (Tables 3 and 5). These data suggest that sufficient RB-C22v

Table 5
Detection of CSFV RB-C22v and BICv in tissues of swine challenged with BICv at 1 and 3 days following RB-C22v infection

Hours post-BICv challenge	Pig #	Tonsil		Mandibular lymph node		Spleen	
		Days post-RB-C22v infection		Days post-RB-C22v infection		Days post-RB-C22v infection	
		1	3	1	3	1	3
6	1	–	–	–	RB-C22v	–	–
	2	–	RB-C22v	–	RB-C22v	–	–
12	1	–	RB-C22v	–	RB-C22v	–	–
	2	–	RB-C22v	–	RB-C22v	–	–
24	1	–	RB-C22v	–	RB-C22v	–	RB-C22v
	2	–	RB-C22v	–	RB-C22v	–	RB-C22v
48	1	–	RB-C22v	RB-C22v/BICv	RB-C22v	–	RB-C22v
	2	RB-C22v/BICv	RB-C22v	RB-C22v/BICv	RB-C22v	–	RB-C22v
72	1	RB-C22v/BICv	RB-C22v	RB-C22v/BICv	RB-C22v	RB-C22v/BICv	RB-C22v
	2	RB-C22v/BICv	RB-C22v	RB-C22v/BICv	RB-C22v	RB-C22v/BICv	RB-C22v

infection and replication in tonsils might preclude BICv replication.

Discussion

Here, we have shown that insertion of a 19 amino acid peptide at the carboxyl terminus of E1 glycoprotein of the CSFV strain Brescia resulted in in vivo attenuation. Unlike the acute fatal disease induced by Brescia and BICv, RB-C22v infection was sub-clinical, characterized by decreased viral replication in tonsils, limited generalization of infection, and reduced virus shedding. Notably, the attenuation observed for RB-C22v was independent of its ability to replicate in primary swine macrophage cell cultures since RB-C22v and BICv exhibited comparable growth characteristics in these cells (Fig. 2A).

The genetic basis and the molecular mechanisms underlying pestivirus virulence remain largely unknown. In CSFV, recent reports have linked specific viral proteins and genomic regions with virulence in pigs (Hulst et al., 2001; Mayer et al., 2004; Meyers et al., 1999; Risatti et al., 2005; van Gennip et al., 2004). A single or double codon mutation abrogating E^{rns} RNAse activity of the CSFV strain Alfort attenuated the virus for pigs (Meyers et al., 1999), as did mutation of the same domain of E^{rns} in BVDV (Hulst et al., 2001). N^{pro} deletion mutants of virulent CSFV strains Alfort/187 and Eystrup were attenuated for pigs (Mayer et al., 2004). More recently, it has been shown that E2 glycoprotein contains genetic determinants for virulence (Risatti et al., 2005; van Gennip et al., 2004). This report describes a novel CSFV virulence determinant associated with the E1 glycoprotein.

While the precise mechanism mediating RB-C22v attenuation in pigs is unclear, it conceivably could involve some aspect of virus attachment and/or entry into critical target cells. E^{rns}, E1 and E2 are structural glycoproteins in the CSFV virion envelope (Weiland et al., 1990, 1999), with E1 and E2 likely anchored to the envelope as both homo and heterodimers linked by disulfide bridges (Thiel et al., 1991; Weiland et al., 1990, 1999) and E^{rns} associated with the virion but lacking the membrane anchor (Weiland et al., 1999). E^{rns} and E2 are known to be involved in virus reception (Hulst and Moormann, 1997), and, more recently, E1 has also been implicated in virus binding and entry (Wang et al., 2004). In silico analysis here predicts that the 19 amino acid insertion at the carboxyl terminus of RB-C22v E1 introduces an alpha helix and turns close to the E1/E2 cleavage site. Such alteration of E1 secondary structure may directly impact a specific virulence determinant directly on E1 or, alternatively, it may affect virulence by altering the ability of E1 to interact with E2 or other viral proteins critical for virus reception, cell tropism, or viral dissemination in the host. The functional significance of E1/E2 heterodimers for reception and infection of target cells in vivo is not known.

Consistent with a possible direct or indirect effect for the altered E1 on virus attachment and/or spread, RB-C22v exhibited a small-plaque phenotype in SK6 cells when compared with parental BICv. Hulst et al. (2000, 2001) have described heparin-sulfate-binding-dependent small plaque variants of CSFV Brescia that contain mutations in E^{rns} following serial passage in SK6 cells. Notably, these CSFV variants had unaltered virulence phenotypes in pigs (Hulst et al., 2000, 2001). With the flaviviruses Japanese Encephalitis Virus (JEV) and Murray Valley Encephalitis Virus (MVEV), passage in cell culture yielded variants with a small plaque phenotype, increased heparin affinity/sensitivity, decreased neurovirulence, and inability to exit peripheral footpad inoculation sites in a mouse model (Lee and Lobigs, 2002). The attenuated flavivirus phenotype was conferred by mutations in the E glycoprotein, analog of the pestivirus E2 glycoprotein (Hurrelbrink and McMinn, 2001). It has been suggested that this flaviviral attenuation may result from increased affinity for glycosaminoglycans, conceivably causing variant viruses to bind cell surface and extracellular matrix proteoglycans to an extent that prevents viremia and spread of the virus to the brain (Lee and Lobigs, 2002); however, attenuated mutants of MVEV demonstrated normal binding and penetration rates, indicating a role for the E protein in virulence at a time subsequent to entry into the host cell (Hurrelbrink and McMinn, 2001). The significance of these observations in flaviviruses for CSFV RB-C22v attenuation remains to be determined.

RB-C22v replication in vivo was limited, including reduced viremia, generalization of infection, and viral shedding, yet, it induced rapid protection from lethal challenge (Tables 2 and 4, Fig. 4) similar to infectivity/protection patterns previously described for other LAV strains of CSFV (Bironi et al., 1987; Terpstra and Wensvoort, 1988). Animals exposed to RB-C22v and challenged with BICv at 3 and 7 DPI were protected against clinical disease, and this was clearly before development of detectable anti-CSFV serum antibodies at 14 DPI. The rapid, solid, and assumedly antibody-independent protection induced by RB-C22v likely involves innate immune mechanisms, e.g. macrophages/monocytes, dendritic cells, and NK cells and their products. Data here indicates that viral interference may also be involved in protection as only RB-C22v was detected in tonsils of pigs protected against challenge with BICv 3 DPI. This lack of BICv in tonsils post-challenge is notable given that primary replication of CSFV normally occurs in tonsils shortly after oronasal inoculation (Bironi and Leunen, 1988; van Oirschot, 1986) (Tables 2, 3 and 5). Consistent with these observations, absence of challenge virus in tonsils of LAV-vaccinated, seronegative pigs challenged with a pathogenic CSFV strain has been shown previously (Bironi and Leunen, 1988). Conversely, comparable amounts of both RB-C22v and BICv were present in tonsils of unprotected pigs challenged 1 DPI (Table 5). Interference with BICv colonization of, or replication in tonsils, could conceivably result from primary replication of RB-C22v, which might induce an antiviral state in tonsils between 1 and 3 DPI that would preclude replication of the virulent challenge virus and progression of the disease (Table 5).

In summary, this study has identified a virulence determinant associated with the E1 glycoprotein of CSFV. This

improved understanding of the genetic basis of CSFV virulence and host range will permit rational design of live attenuated CSF vaccines of enhanced safety, efficacy, and utility.

Materials and methods

Cells and viruses

The SK6 swine kidney cell line (Terpstra et al., 1990) free of Bovine Viral Diarrhea Virus (BVDV) was used throughout this study. SK6 cells were cultured in Dulbecco's minimal essential medium (DMEM) (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) (Atlas Biologicals, Fort Collins, CO). Pathogenic CSFV strain Brescia (obtained from Plum Island Animal Disease Center, Animal and Plant Health Inspection Service, USDA) was propagated in SK6 cells and used for the construction of a cDNA infectious clone (pBIC) from which infectious virus (BICv) was rescued (Risatti et al., 2005). RB-C22v is the E1 mutant virus obtained in this study as described below.

Production of CSFV mutant viruses

Mutants of BICv containing single 57 nucleotide linker inserts were generated using pBIC and a transposon (Tn) mutagenesis kit (Epicentre Technologies, Madison, WI) according to manufacturer's instructions. Briefly, mutant clones containing a Tn insertion were selected in the presence of kanamycin and sequenced using Tn-specific primers to locate genomic position of the insert. The 1.4 kb Tn insertion cassette was removed using *Not*I endonuclease, and linearized clones were religated using T4 DNA ligase (New England Biolabs, Beverly, MA) and transformed into TOP 10 *E. coli* (Invitrogen, Carlsbad, CA). Transformants that grew in the presence of ampicillin but not in the presence of kanamycin were further selected, and the IC plasmids were sequenced with CSFV Brescia specific primers to confirm the position of the remaining 57 nt insert. IC plasmids bearing an insert at desired positions were used to rescue mutant viruses.

Rescue of parental and mutant viruses

Full-length pBIC and mutant IC were linearized with *Srf*I and in vitro transcribed using the T7 Megascript system (Ambion, Austin, TX). Resulting RNA was precipitated with LiCl and transfected into SK6 cells by electroporation at 500 V, 720 Ω, and 100 W with a BTX 630 electroporator (BTX, San Diego, CA). Cells were plated in 12-well plates and 25 cm^2 flasks and incubated for 4 days at 37 °C 5% CO_2 atmosphere. Virus was detected by immunoperoxidase staining using a CSFV E2 specific monoclonal antibody (mAB) WH303 (Edwards et al., 1991). Stocks of rescued viruses were stored at −70 °C. Insert location was confirmed by RT-PCR followed by sequence analysis.

DNA sequencing and analysis

Full-length mutant IC and in vitro rescued mutant viruses were completely sequenced with CSFV specific primers by the dideoxynucleotide chain-termination method (Sanger et al., 1977). Sequencing reactions were prepared with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Reaction products were sequenced on a PRISM 3730*xl* automated DNA Sequencer (Applied Biosystems). Sequence data were assembled with the Phrap software program (http://www.phrap.org), with confirmatory assemblies performed using CAP3 (Huang and Madan, 1999). Protein composition and structure predictions were made using the Genetics Computer Group (GCG) Wisconsin Package versions 7 and 10 software (Devereux et al., 1984).

Western blot analysis

The expression of E1 and E2 in lysates of SK6 cells infected with RB-C22v or BICv was analyzed by Western immunoblots. CSFV E1 was detected with a rabbit antiserum raised against the Brescia strain E1 expressed in a Baculovirus System (BD Biosciences Clontech, Palo Alto, CA). CSFV E2 was detected with mAb WH303. SK6 monolayers were infected (MOI = 1) with RB-C22v or BICv, harvested at 48 h post-inoculation (HPI) using the NuPAGE LDS sample buffer system (Invitrogen), and incubated at 80 °C for 20 min. Samples were run under denaturing conditions in pre-cast NuPAGE 12% Bis–Tris acrylamide gels (Invitrogen). Western immunoblots were performed using WesternBreeze Chemoluminescent Immunodetection System (Invitrogen).

Animal studies

For all animal studies, commercial pigs of 10 to 12 weeks of age and approximately 40 lb in weight were used. To assess virulence, pigs (n = 2) were inoculated intranasally (IN) with 10^5 $TCID_{50}$ with mutant viruses of interest.

For pathogenesis studies, pigs (n = 45) were randomly allocated into three groups and inoculated with BICv (group 1, n = 17), RB-C22v (group 2, n = 26), or mock-infected (group 3, n = 2). Clinical signs (anorexia, depression, purple skin discoloration, excessive lacrimation, staggering gait, diarrhea, and cough) were observed, and body temperature was recorded daily throughout the experiment. Following inoculation, clinical samples (blood, nasal swabs, and tonsil scrapings) were collected, and pigs were sacrificed for tissue collection as follows: group 1, 2 pigs each at 2, 4, 6, 9, and 11 DPI; group 2, 3 pigs each at 2, 4, 6, 9, 11, and 16 DPI; and group 3, 2 pigs at day 0. Tissue samples (tonsil, mandibular and mesenteric lymph nodes, spleen, kidney, bone marrow, and brain) were snap-frozen in liquid nitrogen for virus titration or fixed in 10% neutral-buffered formalin for histopathological studies. Remaining animals from groups 1 and 2 were observed for clinical signs of CSF until day 28.

For RB-C22v protection studies, pigs (n = 24) were randomly allocated into four groups. Pigs in groups 1 and 2 (n = 8) were inoculated with RB-C22v, and animals in groups 3 and 4 (n = 4) were mock-infected. At 7 DPI (groups 1 and 3) or 21 DPI (groups 2 and 4), animals were challenged IN with 10^5 $TCID_{50}$ of BICv. Blood, serum, nasal swabs, and tonsil scrapings were collected at times after challenge. Clinical signs and body temperature were recorded daily throughout the experiment.

For early protection studies, pigs (n = 9) were distributed into three groups of three animals each and inoculated with RB-C22v. At 1, 3, or 5 DPI, all animals were challenged IN with 10^5 $TCID_{50}$ of BICv. Blood, serum, nasal swabs, and tonsil scrapings samples were obtained at times after challenge, and body temperature was recorded daily throughout the experiment.

To determine viruses (RB-C22v or BICv) present in tissues in early protection studies, pigs (n = 25) were randomly distributed in three groups and inoculated IN with 10^5 $TCID_{50}$ of RB-C22v (group 1 and 2, n = 10 each) or 10^5 $TCID_{50}$ of BICv (group 3, n = 5). Groups 1 and 2 were challenged IN with 10^5 $TCID_{50}$ of BICv at 1 or 3 DPI with RB-C22v, respectively. Tissue samples (tonsil, mandibular lymph nodes, and spleen) were obtained (snap-frozen in liquid nitrogen) at 6, 12, 24, 48, and 72 h post-BICv infection, processed for RNA extraction, and analyzed by differential PCR.

Clinical samples

Blood, serum, nasal swabs, and tonsil scrapings were obtained from all animals. Nasal swabs and tonsil scrapings for virus titration were collected with cotton swabs, placed into tubes containing 1 ml sterile D-MEM supplemented with antibiotics, and frozen at −70 °C until used. Peripheral blood was collected in EDTA tubes and used for virus titration and hematologic studies. For serological studies, blood was collected in serum separation tubes. Total white blood cell, lymphocyte, and platelet counts were obtained using a Beckman Coulter AcT (Beckman, Coulter, CA).

Virus titration

Titration of CSFV in clinical samples was performed by endpoint dilution in 96-well plates (Costar, Cambridge, MA) using SK6 cells. After 4 days in culture, viral infectivity was detected by immunoperoxidase assay using the mAB WH303 and the Vectastain ABC kit (Vector Laboratories, Burlingames, CA) (Risatti et al., 2003). Titers were calculated according to the method of Reed and Muench (1938) and expressed as $TCID_{50}$/ml. Primary swine macrophage cell cultures were prepared as described by Zsak et al. (1996).

Serology

Serial two-fold dilutions of serum samples in DMEM (10% v/v FCS, with antibiotics) were added in duplicate to 96-well plates followed by the addition of an equal volume of D-MEM containing 200 $TCID_{50}$ of CSFV strain Brescia. After 1 h of incubation at 37 °C in a moist chamber, approximately 10^3 SK6 cells were added per well. Plates were further incubated at 37 °C and 5% CO_2 for 4 days. Cell monolayers were then fixed with methanol:acetone and CSFV detected by immunoperoxidase staining (see above). Serum neutralization titers were recorded as the reciprocal of the highest dilution of serum that inhibited infection of cells.

CSFV-specific antibodies were detected by ELISA using commercially available kits (HerdChek CSFV Antibody ELISA, Iddex, Westbrook, ME; and CHEKIT CSF-Sero ELISA Test, Dr. Bommeli AG, Switzerland) according to manufacturer's instructions.

Detection of viral RNA

Clinical and tissue samples obtained from inoculated animals were analyzed for the presence of viral RNA by real-time reverse-transcriptase polymerase chain reaction (rt RT-PCR) targeting a region within the 5′-UTR as previously described (Risatti et al., 2003).

Differentiation of RB-C22v from BICv by RT-PCR assay

RT-PCR was performed with total RNA extracted from tissue samples (tonsil, mandibular lymph nodes, and spleen) using the RNeasy kit (Qiagen Inc., Valencia, CA) according to manufacturers instructions. A 10 μl RT reaction [1 μl of MMLV RT buffer, 4 mM dNTPs, 10 pmol of specific reverse primer R3361 (5′AGCTGTCCCTGGGCTCAT3′), 2 μl of total RNA, and 5.3 μl of water] was incubated at 70 °C for 5 min, cooled at room temperature for 20 min, and further incubated for 1 h at 37 °C following addition of 2 units of MMLV RT (Stratagene, Cedar Creek, TX). PCR was performed in a 50 μl reaction [5 μl of 10× Advantage 2 buffer (BD Biosciences Clontech, Palo Alto, CA), 400 μM of dNTPs, 10 pmol of each primer F2379 (5′CCTCATCTGCTTGATAAAAG3′) and R2464 (5′ATGATATTGCGTACCTGT3′), 1 μl of Advantage 2 Polymerase Mix (BD Biosciences Clontech) and 2 μl of cDNA] and included a 95 °C denaturing step for 1 min followed by 25 cycles at 95 °C for 20 s, 52 °C for 20 s, and 68 °C for 30 s, and a final 68 °C extension cycle for 1 min. Amplicons of 168 nt for RB-C22v or 111 nt for BICv were resolved in 2% agarose gels stained with ethidium bromide and visualized under a UV transilluminator.

Immunohistochemistry (IHC)

Tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned. Tissue sections were allowed to adhere to Superfrost/plus slides (Fisher Scientific, Pittsburg, PA), heated for 20 min at 65 °C, and then deparaffinized using xylene. Sections were rehydrated through a graded alcohol series and washed with phosphate-buffered saline (PBS) (pH 7.4). Immunohistochemistry (IHC) was performed as described by Sur et al. (1996). Briefly, 4-

μm sections were first treated with 3% hydrogen peroxide in PBS for 20 min followed by washes in PBS and digestion with 0.05% Protease XIV (Sigma Chemical Co., St. Louis, MO) for 2 min at 37 °C. After several washes with PBS, sections were incubated in blocking solution (5% normal goat serum in PBS) for 30 min at room temperature and then incubated for 2 h at 4 °C with mAB WH303 diluted 1:500 in PBS. Following additional PBS washes, slides were incubated with alkaline-phosphatase-conjugated, goat anti-mouse antibody for 20 min at room temperature.

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